The retinoid-X receptor ortholog, ultraspiracle, binds with nanomolar affinity to an endogenous morphogenetic ligand

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The *in vivo* ligand-binding function and ligand-binding activity of the *Drosophila melanogaster* retinoid-X receptor (RXR) ortholog, ultraspiracle, toward natural farnesoid products of the ring gland were assessed. Using an equilibrium fluorescence-binding assay, farnesoid products in the juvenile hormone (JH) biosynthesis pathway, and their epoxy derivatives, were measured for their affinity constant for ultraspiracle (USP). Farnesol, farnesal, farnesoic acid and juvenile hormone III exhibited high nanomolar to low micromolar affinity, which in each case decreased upon addition of an epoxide across a double bond of the basic farnesyl structure. Similar analysis of the substitution on C1 of methyl ether, alcohol, aldehyde, and carboxylic acid showed that each conferred weaker affinity than that provided by the methyl ester. Attention was thus focused for a ring-gland farnesoid product that possesses the features of methyl ester and lack of an epoxide. A secreted product of the ring gland, methyl farnesoate, was identified possessing these features and exhibited an affinity for ultraspiracle ($K_d = 40$ nM) of similar strength to that of RXR for 9-*cis* retinoic acid. Mutational analysis of amino acid residues with side chains extending into the ligand-binding pocket cavity (and not interacting with secondary receptor structures or extending to the receptor surface to interact with coactivators, corepressors or receptor dimer partners) showed that the mutation C472A/H475L strongly reduced USP binding to this ring gland product and to JH III, with less effect on other ring-gland farnesoids and little effect on binding by (the unnatural to *Drosophila*) JH I. Along with the ecdysone receptor, USP is now the second arthropod nuclear hormone receptor for which a secreted product of an endocrine gland that binds the receptor with nanomolar affinity has been identified.

Developmental decisions in invertebrates are regulated by steroids [1,2] and terpenoid-derived farnesoids (i.e. methyl farnesoate, juvenile hormones) [3,4]. The vertebrate retinoid-X receptor (RXR) can bind to 9-*cis* retinoic acid (RA; $K_d = \sim 20$ nM) [5], as well as dietary chlorophyll-derived phytanic acid ($K_d = 2.3$ μM) [6], in addition to several long-chain unsaturated fatty acids (e.g. docosahexaenoic acid, $K_d = 66$ μM) [7]. Vertebrate RXR and RA-related compounds continue to yield new insights into regulatory mechanisms.

However, the understanding of those RXR mechanisms is far ahead of that for the invertebrate ortholog of RXR (ultraspiracle; USP) and insect terpene-derived farnesoids.

Starting from the original model of a single RA receptor for a single RA ligand, it was determined that there is more than one form of active RA *in vivo*, including epoxidized forms, hydroxylated forms and geometric isomers [8], as well as an esterified form for which a specific esterase has recently been cloned [9]. Various such derivatives of the parent all-*trans* RA were found to bind to retinoic acid receptor (RAR), some as strongly as all-*trans* RA [10]. In transfection assays, various of these RA forms activated RARα, RARβ or RARγ with differing relative activities depending on the receptor, in some cases exhibiting greater activity than all-*trans* RA [11]. Some of these forms of RA were able to modulate position specificity in the embryo [12] and exhibited activities *in vivo* as strong as those seen with all-*trans* RA [13].

Subsequent studies also showed that there is more than one type of RA receptor: RXR, RAR and ROR are all capable of binding RA(s) [14–16]. Furthermore, each of these different types of RA receptors has very different affinity relationships to the different ligands, e.g. RAR binds both 9-*cis* and all-*trans* RA with nanomolar affinity, whereas RXR can bind only 9-*cis* RA with such nanomolar affinity. Hence, one nuclear receptor (e.g. RAR) functioning to bind with high affinity and be activated by the all-*trans* form of RA is a different matter than a different receptor (RXR) that binds with high affinity and is activated by another form of RA (9-*cis*), both of which are a different matter than ROR binding to and being antagonized by all-*trans* RA.

The above principles appear to apply to arthropods as well. In the crustaceans, the mandibular organ produces the terpenoid ester methyl farnesoate [17,18]. In insects, this same compound methyl farnesoate is produced in the glands (corpora allata) of exopterygote insects [19], and there is also recent evidence of its production in the corpora allata of endopterygote Lepidoptera [20]. Several independent studies have confirmed the production of methyl farnesoate from the larval ring gland of higher (calypterate) Diptera [21], and from the corpora allata of adult calypterate Diptera [21,22]. As with vertebrates and RA, several hydroxylated [23–25] and epoxidized variations in the structure of methyl farnesoate have been reported, as has a specific esterase that hydrolyzes the methyl ester (e.g. Campbell *et al.* [26] for specific esterase in *Drosophila melanogaster*). In the case of higher Diptera, the synthetic glands secrete methyl 10,11-epoxy-farne-soate (juvenile hormone III; JH III) and in some species possibly also the methyl-6,7-epoxy-farnesoate [27]. The dipteran ring gland/corpora allata appear unique in also secreting bisepoxyJH III [28].

With respect to potential receptors for terpene-derived ligands in invertebrate systems, RXR has been cloned from sponge and jellyfish. Although 9-*cis* RA did not bind to the purified recombinant receptor of the former, it did bind to the latter at low nanomolar concentrations, however, it did not transactivate 9-*cis* RA signaling via that receptor in a cell transfection system [29,30]. Recently, RXR from mollusc-bound 9-*cis* RA at 1 μM did transactivate in a cell-transfection assay [31]. Very similar RXR has also been reported from crustaceans and arachnids [35,36], but in neither case did the recombinant receptor bind 9-*cis* RA or transactivate 9-*cis* RA signaling in a cell-transfection assay. RXR has also been cloned from exopterygote insects such as locust, where the receptor did not bind radiolabeled insect JH III [32]. In two endopterygote orders, Diptera and Lepidoptera, there has been such divergence in the RXR sequence that it has the special name ultraspiracle (USP) [33]. A different question from the function of invertebrate RXR/USP is the identity of *per se* receptors for the epoxidized forms of methyl farnesoids (juvenile hormones). As reviewed previously, several cellular proteins are reported to physically bind JHs, including the MET protein [34,35], an ovarian membrane protein [28] and USP [36,37].

It is well established in the field of vertebrate orphan nuclear receptors that a necessary stage of experimental inquiry is to develop evidence-based hypotheses on the structural features that might be possessed by potential endogenous ligands. One conventional approach used to develop such structural hypotheses is a systematic analysis of the effect of altering specific moieties on the affinity of binding to the receptor. A second common experimental objective, which is subserved by the above experimental approach, is the identification of lead structures toward commercial compounds or experimental probes that agonize or antagonize the target receptor. In fact, it is well-established in the nuclear receptor field that the development of useful synthetic agonists/antagonists can occur before the endogenous ligand(s) of the receptor are known [38].

Several structure–activity studies have been performed on the heterodimer partner of USP (i.e. the ecdysone receptor; EcR), for the purpose of developing commercially viable insecticides or experimental probes. However, no similar systematic structure–binding activity study for USP that explores chemical

features that impart stronger vs. weaker binding of a chemical structure to USP has been published. The study reported here was performed not to identify the JH receptor, but rather for the above purposes, of identifying chemical features that impart higher affinity chemical binding to USP, to aid in: (a) prompting test-able hypotheses in future investigations on potential endogenous ligand(s) for USP, and (b) identifying compounds with potential commercial insecticidal properties as USP agonists/antagonists or compounds that would be useful as experimental probes in the dis-covery of new USP-dependent pathways. Given that vertebrate RXR and *Drosophila* USP are evolutionary orthologs, and that the closest known chemical struc-tures in *Drosophila* to vertebrate RA are products of the farnesoid biosynthesis pathway, this study analyzed both natural and synthetic variations of the farnesoid scaffold. We report the identification of a natural farnesoid product of the ring gland with affinity for USP comparable with the affinity of 9-*cis* RA for RXR.

Results

Analysis of components of the farnesoid biosynthesis pathway

The dipteran ring gland synthesizes farnesol in a ter-pene biosynthesis pathway starting from acetate. We observed that incubation of USP with farnesol did not significantly reduce USP fluorescence, even at 100 μM. This may mean that farnesol does not bind a signifi-cant portion of the receptor preparation at that con-centration, or that it does bind to the pocket, but not in a way that quenches receptor fluorescence. Hence, we tested whether farnesol could competitively displace a quenching ligand (JH III) from the receptor, so as to relieve the receptor quenching caused by JH III. That is, if the nonfluorescence-suppressing ligand can actu-ally bind, then as it displaces a fluorescence-suppres-sing ligand, the suppression induced will be relieved. As shown in Fig. 1A, farnesol could bind to USP, competitively displacing JH III, with a K_i of ~ 5 μM, relieving the suppression in fluorescence that would otherwise be caused by JH III. Hence, farnesol can be shown to bind to USP.

In the farnesoid biosynthesis pathway of the ring gland, farnesol is converted to farnesal (by as yet unidentified putative dehydrogenases) [39]. We observed farnesal binding to USP and suppressing its fluorescence, with an affinity constant of $K_d = 700$ nM (Fig. 1C), which is much stronger that the affinity exhibited by farnesol. The next step in the biosynthesis

pathway is the conversion of farnesal to farnesoic acid (again by an unknown putative dehydrogenase). Farnesoic acid exhibited a weaker affinity for USP than the aldehyde, with an affinity constant of $K_d = 3$ μM (Fig. 1E). The farnesoid biosynthesis pathway of most insects is considered to lead to a secreted prod-uct, JH III (the methyl esterified, epoxidized product), which is also a secreted by the dipteran ring gland. We found a USP affinity constant of 7 μM for JH III (Fig. 1H).

### Effect of epoxidation on the affinity for USP

Because the epoxide group at C10–C11 is a hallmark of the JH product of farnesoid biosynthesis, we tested the effect of epoxidation on the affinity of the above farnesoid compounds for USP. Epoxidation of the C10–C11 olefin of farnesol decreased the affinity by at least 10-fold ($K_i > 50$ μM; Fig. 1B) (epoxyfarnesol did not significantly suppress USP fluorescence). C10–C11 epoxidation of farnesal also significantly weakened its affinity for USP ($K_d = 12$ μM; Fig. 1D). Similarly, epoxidation of farnesoic acid (to yield JH III acid) weakened the affinity of the farnesoid for USP ($K_i = 10$ μM; Fig. 1F), and epoxidation of methyl farnesoate also strongly decreased the affinity constant ($K_d = 7$ μM; Fig. 1H). Finally, a second biosynthetic end product of the farnesoid pathway in the dipteran ring gland is formed by the addition of a second epoxide group to the C6–C7 olefin of JH III, to yield bisepoxy JH III. That second epoxidation yields a product (bis-epoxy JH III) with such weak binding to USP that the affinity was difficult to measure ($K_d > 50$ μM; Fig. 1I). These results provide strong evidence that high-affinity binding by a farnesoid structure to USP requires the absence of epoxidation at C10–C11 or C6–C7 along the farnesoid backbone (Table 1).

Effect of methyl esterification

We also analyzed the effect of the nature of the substi-tutions at C1 on the affinity for USP, i.e. a methyl ether (Fig. 2G), an alcohol (Fig. 1A), an aldehyde (Fig. 1C), or a carboxylic acid (Fig. 1E). The more polar alcohol and carboxylic acids, and the less polar methyl ether, all conferred weaker binding than did the aldehyde (Table 2).

High-affinity natural farnesoid products

The data indicated that a farnesoid with high affinity for USP would be not epoxidized and would possess a polarity on C1 nearer to that of an aldehyde, being
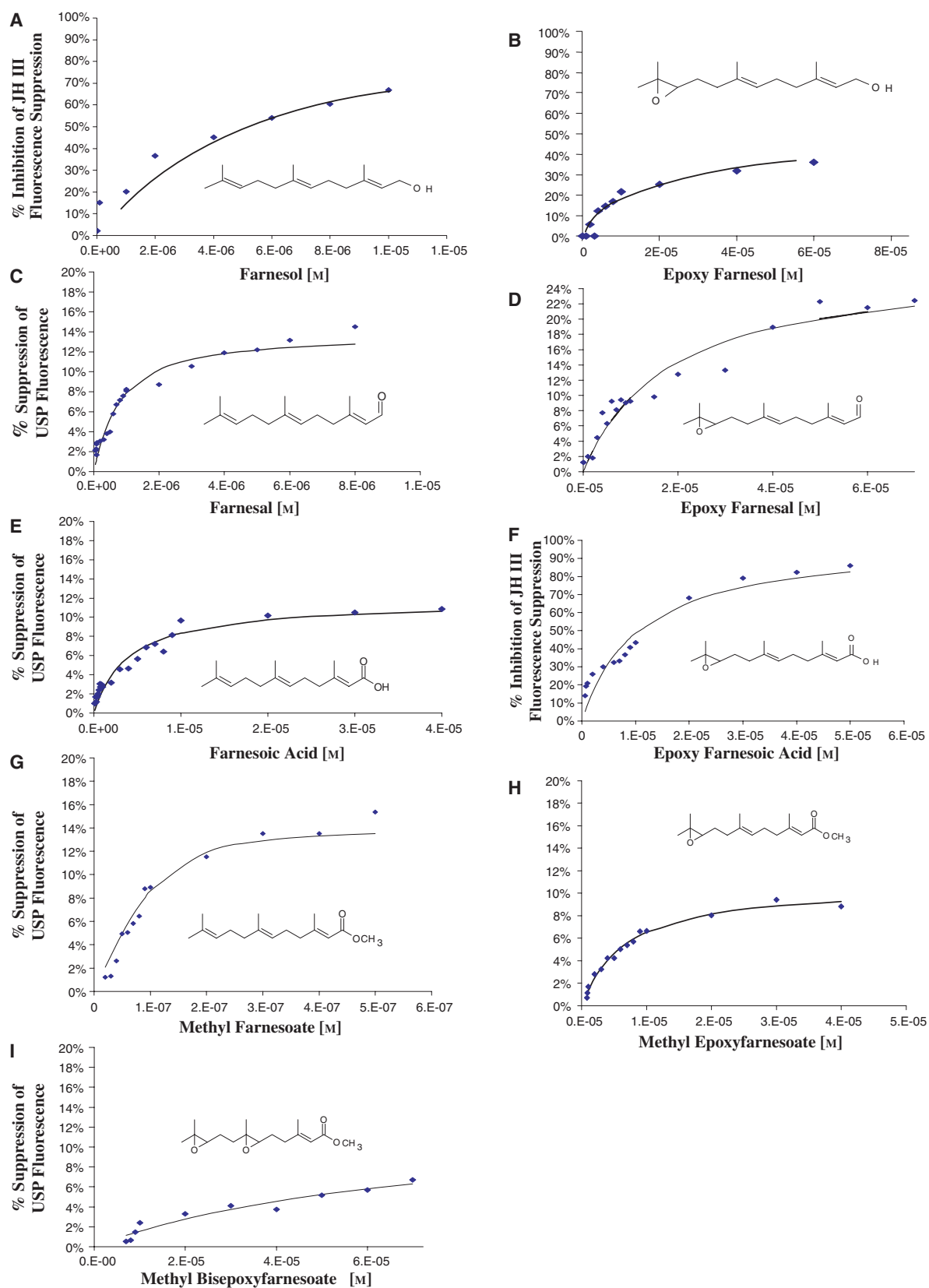
Table 1. Comparison of the affinity constants of natural ring-gland farnesoids and their epoxidized counterparts. The averages are based on three or more replications, except for epoxyfarnesoic acid and farnesol, which are each based on two replications. The affinity constant for epoxy farnesol could not be determined because the affinity was so weak that it required concentrations over 50 µM, which presented technical problems with the assay. Values in parenthesis are K_i, as determined by competition binding assay using methyl epoxyfarnesoate as the primary ligand (30 µM).

Compound	Avg. K_d (M)	SD	Effect of epoxy group on affinity to dUSP
Farnesol	(5.3×10^{-6})	1.1×10^{-6}	
Epoxy farnesol	$(> 5.0 \times 10^{-5})$		↓
Farnesal	5.8×10^{-7}	1.5×10^{-7}	
Epoxy farnesal	1.2×10^{-5}	7.0×10^{-7}	↓
Farnesoic acid	3.4×10^{-6}	8.5×10^{-7}	
Epoxy farnesoic acid	(9.9×10^{-6})	1.5×10^{-5}	↓
Methyl farnesoate	4.4×10^{-8}	2.5×10^{-8}	
Methyl epoxyfarnesoate	6.7×10^{-6}	2.4×10^{-6}	↓
Methyl bisepoxyfarnesoate	1.1×10^{-4}	5.0×10^{-5}	↓

more polar than the methyl ether but less polar than the alcohol or carboxylic acid. A farnesoid biosynthetic product of the ring gland with these properties is the ester, methyl farnesoate. It is of great significance that methyl farnesoate exhibited the highest affinity of all the tested compounds for USP, with a $K_d = 40$ nM (Fig. 1G). This affinity of USP for methyl farnesoate is very similar to that of RXRα for 9-*cis* RA [5]. Methyl farnesoate is also one of the three known secreted products of the *Drosophila* ring gland [21].

Although production of higher homologs possessing ethyl (rather than methyl) side branching at C11 (JH II), C11 and C7 (JH I), or C11, C7 and C3 (JH 0) is not currently known from *Drosophila*, JH I has been reported to be synthesized by a nonring gland tissue (male accessory gland) in mosquitoes [38]. Intriguingly, although the affinity of USP for JH I was much weaker than that for methyl farnesoate, it was several fold stronger ($K_d = 1.6 \pm 0.6$ µM; Fig. 2A) than for JH III ($K_d = 7$ µM; Fig. 1H). However, unepoxidized JH I (i.e. JH I counterpart to methyl farnesoate; compound **4**; Fig. 3) bound dUSP 1000× more strongly ($K_d = 1.6 \pm 0.3$ nM; Fig. 2H) than did epoxidized JH I, further confirming that epoxidation of the molecule decreases affinity. In addition, the affinity of unepoxidized JH I for dUSP was stronger than that of methyl farneosoate. Further adjustment of the isomeric configuration of JH I increased the binding affinity even more (D. Jones, G. Jones and D. Coy, unpublished data).

Pocket mutational analysis of ligand interaction with USP

Given the above results, we sought to test *in vivo* whether a ligand-binding function for USP is necessary. This approach necessitated that we first identify a mutation that would reduce binding by those known secreted products of the ring gland with the highest affinity to dUSP: methyl farnesoate (the strongest binder) and JH III (which with low micromolar binding could begin to load USP if a local tissue concentration of JH III reached high nanomolar levels). The recently published crystal structure of methoprene acid in complex with RXRβ showed the distal end of the ligand (i.e. C12 end) in contact with residues corresponding to C472 and H475 in dUSP [40]. In earlier studies, using the assumption that α helix 3 in USP adopts *in vivo* a conformation similar to that of RXRα, Sasorith *et al.* [41] docked JH III with the USP ligand-binding pocket using computer-modeling techniques, and postulated that JH III may be in contact with C472 in the dUSP. Therefore, we mutated the two residues C472 and H475 to alanine and leucine, respectively. As shown in Table 2, this mutation most strongly reduced (by 80–90%) the K_d for methyl farnesoate and JH III, with less effect on the two nonsecreted farnesoid products of farnesal and farnesoic acid. Interestingly, this mutation had little significant effect on the binding constant for JH I (which further

Fig. 1. Saturation binding curves for natural ring-gland farnesoids, and their epoxy derivatives, in binding with wild-type USP from *Drosophila melanogaster*. *Y*-axis is the percent suppression of intrinsic dUSP fluorescence caused by the indicated compound, except for three cases in which the values indicate the percent of JH III-induced suppression that is competitively relieved by the indicated compound (A, B, F). (A) Farnesol, (B) epoxyfarnesol, (C) farnesal, (D) epoxyfarnesal, (E) farnesoic acid, (F) epoxyfarnesoic acid, (G) methyl farnesoate, (H) methyl epoxyfarnesoate (JH III), (I) methyl bisepoxyfarnesoate (bisepoxyJH III).

A — % Suppression of USP Fluorescence vs. Juvenile Hormone I [M], wt USP

B — % Suppression of USP Fluorescence vs. Juvenile Hormone I [M], C472A/H475L USP

C — % Suppression of USP Fluorescence vs. Methyl Farnesoate [M], C472A/H475L USP

D — % Suppression of USP Fluorescence vs. Methyl Epoxyfarnesoate [M], C472A/H475L USP

E — % Suppression of USP Fluorescence vs. Farnesal [M], C472A/H475L USP

F — % Suppression of USP Fluorescence vs. Farnesoic Acid [M], C472A/H475L USP

G — % Suppression of USP fluorescence vs. Farnesyl Methyl Ether, wt USP

H — % Suppression of USP fluorescence vs. Methyl 14, 15 di-methyl Farnesoate, wtUSP

Fig. 2. Saturation binding curves for selected farnesoids with wild-type or mutant C472A/H475L dUSP. *Y*-axis is the percent suppression of intrinsic dUSP fluorescence caused by the indicated compound. (A, B) JH I binding to wild-type and mutant dUSP, respectively. (C, D) Methyl epoxyfarnesoate (JH III) binding to wild-type and mutant dUSP, respectively. (E) Farnesal binding to mutant dUSP. (F) Farnesoic acid binding to mutant dUSP. (G) Farnesyl methyl ether binding to wild-type dUSP. (H) Methyl 14,15-dimethyl farnesoate binding to wild-type dUSP.

Table 2. Comparison of the effect on affinity of either the various indicated (by underlining) substitutions on the C1 position of the ligand, or of the mutation C472A/H475L to the receptor (USP).

Compound	Ratio K_d (test compound) to K_d (methyl farnesoate)	Ratio K_d (mutant C472A/H475L) to K_d (wild-type dUSP)
Farnesyl methyl ether	250	–
Farnesol	125	–
Farnesoic acid	77	2.1
Farnesal	13	2.4
Methyl farnesoate	1	4.1
Methyl epoxyfarnesoate	152	5.7
Methyl 14,15-dimethyl-epoxyfarnesoate (JH I)	44	0.9

supports that the mutation did not globally disrupt USP tertiary structure; Fig. 2A,B, Table 2).

Discussion

Implications for models of USP biochemical function

The field of nuclear hormone receptors has considered for many years the status of USP as an orphan receptor. Since discovery of the EcR, extensive biochemical studies on EcR ligand binding have fostered detailed models of EcR action that expressly provide for the *in vivo* necessity of the EcR ligand [1]. Only recently has the hypothesis of necessary ligand binding by EcR been directly tested by assessing the ability of a mutant EcR (with *in vitro* loss of 20OH-ecdysone binding) to rescue (or not) the *in vivo* the lethal null EcR phenotype [42].

In an exciting recent development using cell-free physical biochemistry, the insect nuclear receptor E75 was found to have the capacity to bind NO or CO to a heme center, in a dynamic equilibrium, and this binding under cell-free conditions physically affected the interaction of E75 with the AF2 motif fragment of its (*in vivo*) heterodimer partner, DHR3 [43]. Although the affinity of E75 heme for NO was not measured, and the local intracellular concentration of NO undetermined, and therefore the potential for *in vivo* NO modulation of E75 being kinetically inestimable, the

authors correctly observed that the ability of E75-heme to bind NO and CO, and for these gases to modulate cofactor binding and transcriptional activity (at a NO donor concentration of 200 μM), suggests a role in mediating NO and/or CO intercellular signaling. In the context of the *in vivo* parameter of the local tissue concentration of NO, the importance has been emphasized of these physical binding and cell transfection assay data, which show that is E75 is physical structured with the features necessary for participation in gas signaling, enabling the inference that E75 is powered by gas ligand [44].

We previously reported that dUSP (which had been postulated to not possess the capability of binding ligand in dynamic equilbrium) can physically bind in such a kinetic manner to JH III [37]. We also reported that such binding promotes or stabilizes the dUSP homodimer, and can also cause repositing of its AF2 motif [45,46]. In addition, in a cell-transfection model system, dUSP has the ability, via its ligand-binding pocket, to transduce transcriptional activation by exogenous JH III [46]. As with NO, the local tissue concentration of the three secreted farnesoid products of the *D. melanogaster* ring gland (JH III, bisepoxy JH III, and methyl farnesoate) are unknown, and hence on a kinetic basis JH III as an *in vivo* ligand for dUSP cannot be demanded or dismissed. However, these physical binding and cell-transfection assay data show that dUSP is physically structured with the features necessary for transducing signaling from a ligand that binds in dynamic equilibrium. The farnesoid products of the ring gland being the closest known endogenous products in *Drosophila* to the vertebrate RA, and USP being the *Drosophila* ortholog of vertebrate RXR, we have taken our experimental inquiries in this study to the next step of systematic exploration of the features of a farnesoid scaffold that impart the higher binding affinities to dUSP. The outcome of these studies, from the compounds tested, is that the dUSP ligand-binding pocket favors the absence of epoxidation, the presence of a methyl ester, and the product of the farensoid pathway of the *Drosophila* ring gland to which it has the strongest (nM) affinity is the secreted product, methyl farnesoate. It seems prudent that models of potential function of USP *in vivo* account for these data. (These results complement our earlier
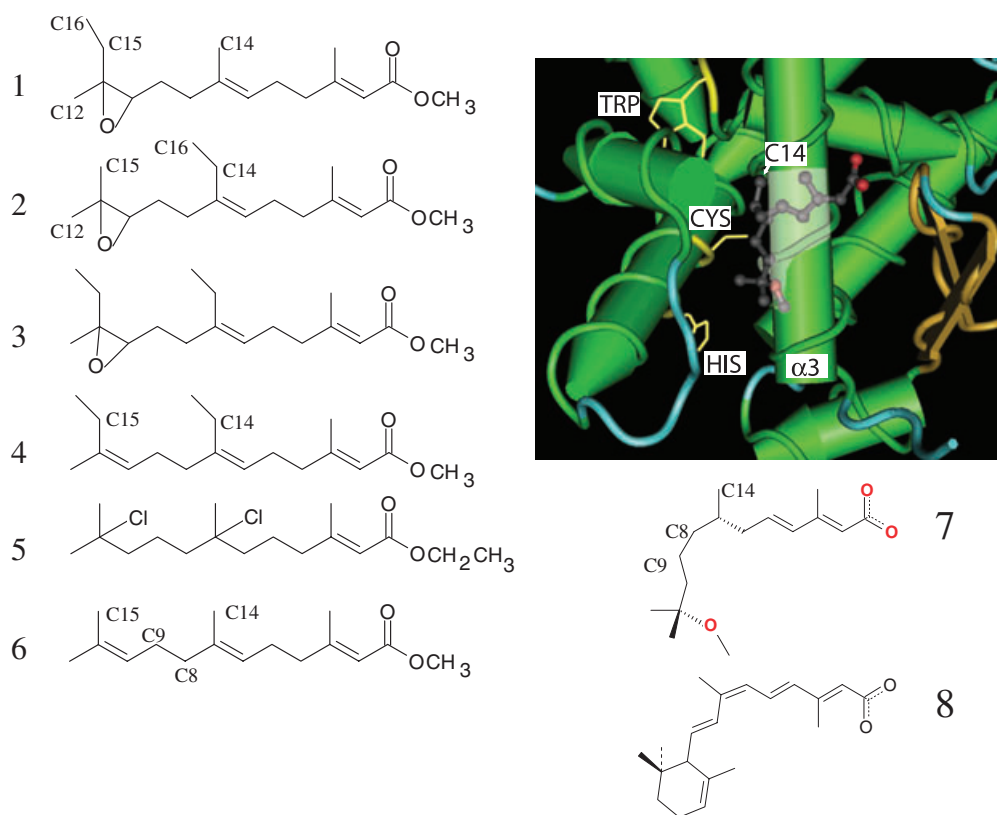
Fig. 3. Structural features of various farnesoid derivatives. Compounds shown are (1) JH II; (2) isoJH II; (3) JH I; (4) methyl 14,15-dimethyl farnesoate; (5) methyl C7,C11-dichlorofarnesoate; (6) methyl farnesoate; (7) methoprene acid, oxygen atoms in red, and diagrammatically illustrating the right angle bend of the methoprene acid arising from the flexibility of the single bonds around C7 and C8; (8) 9-*cis* retinoic acid. The inset panel shows the position of methoprene acid in the ligand-binding pocket of RXRβ, as projected using the crystal structure coordinates published by Svennson *et al.* [40], using CND3 software. The C14 of the ligand is expressly labeled, and the oxygen atoms shown in red. Also shown in yellow are the side chains of three conserved amino acid residues that extend into the ligand-binding pocket, corresponding to tryptophan 305, cysteine 472 and histidine 475 in dUSP. A portion of alpha helix 3 (α3) of the RXRβ is shown semitranspar-ently to visualize the portion of the ligand otherwise blocked from view.

reports that, individually, methyl farnesoate and JH III exert a similar amount of fluorescence suppression to USP [45], which is harmonious with our earlier obser-vation that addition of methyl farnesoate to an already saturating level of JH III does not further decrease the level of fluorescence suppression [37].)

Implications on signaling function of methyl farnesoate

Richard *et al.* [21], showed that the larval dipteran cor-pora allata/ring glands secrete not only at least two forms of epoxidized methyl farnesoids (JH III and bis-epoxy JH III), but also methyl farnesoate itself. At times methyl farnesoate production was detected at much higher rates than JH III. Perhaps increasingly significant when juxtaposed with our results is that

although the biosynthesis studies showed only trace production of methyl farnesoate by adult female cor-pora allata (up to 98% being bisepoxy JH III), earlier during the mid third-larval instar more methyl farneso-ate was shown to be secreted by the ring gland than JH III. In fact, at the earliest larval time point pub-lished (early third instar), the production of methyl farnesoate is almost equal that of bisepoxy JH III (and at which developmental time JH III, the only form for which the measured third instar hemolymph JH titer is based, is but a comparative trace of the biosynthetic output of the third-instar ring gland) [21]. The pres-ence of methyl farnesoate in its particular natural blend with JH III and bisepoxyJH III restores oogen-esis to allatectomized adult *Phormia regina* [22]. Our results stimulate new thinking as to potentially mor-phogenetically active secretion(s) of the ring gland

during early larval dipteran development, and urge new assessment of long neglected, as yet unexplained data of earlier endocrine researchers such as Vogt and Bodenstein.

Implications for understanding how farnesoids fit into the USP ligand-binding pocket

The natural ligands for nuclear receptors do not fully occupy the cavity of the ligand-binding pocket. For example, 53–67% occupancy occurs for 9-*cis* RA (in RXRβ), vitamin D3 (in vitamin D receptor), 9-*cis* RA (in RXRα), estradiol (in estradiol receptor), all-*trans* RA (in RARγ), and progesterone (in progesterone receptor). The unoccupied space provides an opportunity to design synthetic ligands that extend into the space and make new or stronger contacts with the residues lining the binding pocket, increasing the binding affinity of the receptor for the synthetic ligand compared with the natural ligand. For example, in an effort to identify ligands that would distinguish between RARα and RARγ, it was noted that the methionine residue M272 in RARγ protrudes into space that is not occupied by the corresponding isoleucine I270 in RARα. It was postulated that a synthetic ligand that extends into that empty space in RARα would enable selectivity for binding to RARα, but not RARγ, and such a synthetic ligand was in fact identified (BMS614) [47]. Similarly, the crystal structure of RXRα in complex with 9-*cis* RA [48] shows unoccupied space close to W305 (W318 for dUSP), to which the closest approach by 9-*cis* RA is the methyl side branch C19 (C14 for farnesoids). The authors postulated that synthetic 9-*cis* RA analogs with extension into that unoccupied space could yield new and more specific ligands.

Similarly, unoccupied space in this area may also remain for dUSP when it is in complex with methyl farnesoate or related JHs. If that is the case, then a methyl farnesoid derivative with a larger substituent on C14 which extends further into that space could yield a higher affinity ligand. In that regard, it is interesting to note that Postlethwait [49] tested in the *Drosophila* white puparia bioassay a methyl epoxyfarnesoate derivative in which the C14 methyl branch was replaced by a longer ethyl branch (compound **2**, Fig. 3). The compound can be considered as an isomer of JH II (iso-JH II), in which the longer ethyl side branch on the distal end of JH II (compound **1**, Fig. 3) is moved to the farnesoid mid-backbone to replace C14 methyl group. Intriguingly, although in that bioassay JH II is weaker than either JH I or JH III, the synthetic iso-JH II exerted more biological

activity than JH I, JH II, or JH III. The same trend for higher activity by iso-JH II in bioassay on *Tenebrio molitor* has been reported [50].

These *in vivo* results with iso-JH II may relate to the behavior of JH I, JH II, and JH III in physical binding to USP. We have previously shown that JH II binds to the dUSP ligand-binding pocket in a qualitatively different manner than JH I or JH III – it has little if any affect on USP fluorescence, whereas JH I and JH III suppress USP fluorescence [51]. The only structural difference between JH II (compound **1**, Fig. 3) and JH III (Fig. 1A) is that for the C15 methyl branch near the distal epoxide group, which is possessed by JH III, JH II has a longer ethyl branch. This same C15 methyl branch end on the distal end of the ligand in the RXRβ–methoprene acid crystal complex is near the opening of the ligand-binding pocket (Fig. 3). The C15 methyl branch interacts with the equivalent of residues C472 and H475 in dUSP (i.e. not near tryptophan residue W305, which is deep in the pocket). It could be that the longer ethyl group on the distal end of JH II alters the position of JH II into the pocket space of dUSP relative to the positive of JH III, such that the approach of the mid-backbone C14 methyl side branch to the tryptophan residue (W305) is altered for JH II, perhaps moved further away, and hence in binding to dUSP JH II has little effect on fluorescence from W305.

In contrast to JH II, JH I behaves similarly to JH III in the binding assay, insofar that each suppresses fluorescence to a similar extent [51]. JH I and JH III are also more similar in terms of activity in the *Drosophila* white puparia assay, each producing similar dose–response effects [45,51]. In fact, in our binding assays, JH I (not natural to *Drosophila*) shows several fold stronger binding to *Drosophila* USP than does *Drosophila* JH III (Table 2). Thus, even though JH I possesses the same disfavoring (as JH II) conversion of the C15 methyl to an ethyl branch, it has one additional distinction from JH III that both moves its USP-binding activity back toward that of JH III and confers on it a biological activity similar to that of JH III. That is, JH I also possesses the same conversion of the mid-backbone C14 methyl branch to a longer ethyl branch as seen in the highly active iso-JH II [49].

As mentioned above, the crystal structure of RXRβ in complex with methoprene acid showed the C12 end of the ligand to be in contact with residues corresponding to C472 and H475 in dUSP [40]. Using computer-modeling techniques, Sasorith *et al.* [41] docked JH III with the USP ligand-binding pocket and inferred that JH III may be in contact with the equivalent of C472 in the dUSP. Our results, using different

methods, support that inference. We found that mutation of these residues decreased the physical binding by methyl farnesoate and JH III more than binding by other related non-JH farnesoids. In addition, preliminary experiments suggest that the mutant receptor (C472A/H475L) could not fully supply the missing function of wild-type USP in null *usp*[2] flies during development, which is consistent with an *in vivo* ligand-binding function for USP that involves either or both of these residues (D. Jones, G. Jones and R. Thomas, unpublished data).

We anticipate that the mechanism by which dUSP shows a physical response to methyl farnesoate binding is different to than that upon JH III binding. We previously tagged the transcription-modulating AF2 group of the ligand-binding domain using a pair of reporter tryptophan residues [51]. Upon binding of JH III, the AF2 module was induced to reposition in such a way that net receptor fluorescence increased rather than decreased. However, binding by methyl farnesoate did not have the same effect, but rather USP fluorescence decreased to a similar extent as for wild-type USP. That is, methyl farnesoate and JH III cause different conformational outcomes to USP. Whereas JH III exerts an effect upon USP conformation with the outcome of transcriptionally activating the receptor [45,46,52], methyl farnesoate may exert a different regulatory effect via USP. In preliminary Sf9 cell transfection experiments, using model promoters from the JH esterase gene and several hexamerin genes, in certain contexts JH III and methyl farnesoate do not exert the same transcriptional effects on activation of the model promoter (G. Jones, D. New and G. Andruszewska, unpublished data).

Implications for the design of synthetic USP-binding compounds

Since the late 1960s efforts to identify commercially viable third-generation insect-selective pesticides with JH action have proved expensive, laborious and time consuming, and have yielded only a few JH agonists [53,54]. Furthermore, there is not a single commercially marketed anti-JH compound, which if found would have tremendous implications for those agricultural crops in which the larval insect is the commercially damaging life stage [53]. There is a very clear reason for this lack of progress: lack of a cloned nuclear receptor that binds to any of the secreted methyl farnesoid hormones. Hence, most work on commercial compounds targeting specific insect nuclear hormone receptors has been performed on the (cloned since 1991) EcR, which functions as a heterodimer with

USP. In more recent years, two cloned receptors have been identified, for which the recombinant receptor in biochemical demonstration binds to methyl epoxyfarnesoate (JH III): the MET protein [55] and USP. However, until our results, no cloned receptor has been reported with high (nM) affinity binding to methyl farnesoate. Unepoxidized JH I, a compound foreign to *D. melanogaster*, was shown to possess an affinity for dUSP greater than any of the known endogenous farnesoids that we tested. Hence, our results establish a proof of concept that, using a farnesoid scaffold, compounds foreign to *D. melanogaster* can be identified that bind to dUSP more tightly than potential endogenous ligands. Our data identify USP as a potential practical target for selective, high-affinity compounds based on a methyl farnesoid structure.

Experimental procedures

Chemicals

Potential ligand structures used in these studies and their sources were: methyl epoxyfarnesoate (JH III), farnesol and farnesal from Sigma-Aldrich (St Louis, MO; farnesal was also synthesized by PT, see below); JH I from SciTech (Prague, Czech Republic); methyl farnesoate and farnesoic acid from Echelon Biosciences (Salt Lake City, UT); farnesyl methyl ether from Fluka (St Louis, MO); epoxy farnesol, epoxyfarnesal, epoxyfarnesoic acid (JH III acid), and methyl bisepoxyfarnesoate (bisepoxyJH III) were synthesized as described below. Each of these was prepared as a stock solution in ethanol, and was dispensed into the 1.5 mL binding reactions to their respective concentrations, with a final ethanol carrier concentration of 0.1%.

Chemical syntheses

All chemicals used for syntheses including (E,E)-3,7,11-trimethyl,2,6,10-dodecatrien-1-ol (farnesol) were purchased from Sigma-Aldrich and were of the highest purity available. Solvents were GC-MS grade from Burdick and Jackson (Muskegon, MI) and 18 mΩ water was obtained from a Milli Q UVplus system. MSl analysis was performed using chemical ionization as described by Teal *et al.* [56] and electron impact spectra were obtained using the same instrument operated at 70 eV with a filament bias of 11 765 mV. NMR spectra were obtained from material dissolved in CDCl$_3$ using a Bruker AC-300 instrument equipped with a 5 mm probe. One-dimensional spectra were from 64K data points. The operating frequency was 300.14 MHz for ^{1}H and spectral width was set at 6024 Hz. The number of scans acquired was 32.

Farnesal was prepared from farnesol by oxidation with a molar excess of activated MnO$_2$ (Sigma-Aldrich) in hexane

as described by Corey *et al.* [57]. After filtration the reaction mixture was applied to a 20 × 2.5 cm (i.d.) glass column slurry packed with silica in hexane. The column was eluted sequentially with 10, 20 and 30% diethyl ether in hexane (250 mL each). Farnesal eluted in the 20% ether fraction as indicated by GC-MS. The aldehyde was further purified by HPLC using a using a Rheodyne 7125 injector, a Kratos Spectraflow 400 pump and Waters 410 differential refractometer using an Adsorbosil silica column (250 × 10 mm i.d. 10 m particles) eluted with 20% ethyl acetate in hexane (flow = 5 mL·min^{-1}). MSl analysis indicated the aldehyde fraction to be 98% pure and free of farnesol.

Methyl farnesoate was prepared from farnesal by the addition of the aldehyde (1 g) drop-wise to a mixture of NaCN (400 mg) acetic acid (1 g), activated MnO$_2$ (2 g) and 250 mL methanol. The mixture was stirred overnight at room temperature [57]. The mixture was filtered, diluted by the addition of 250 mL water and extracted three times with 500 mL hexane. The organic extract was concentrated prior to HPLC separation as described above. Analysis of methyl farnesoate so obtained indicated purity of 98% and the absence of farnesal.

Monoepoxides of farnesal, farnesol and methyl farnesoate were prepared by drop-wise addition of the individual starting materials, dissolved in benzene, into a 1.25 M excess of *m*-chloroperbenzoic acid in dry benzene [58]. The mixture was stirred overnight prior to dilution with 250 mL hexane, neutralization by shaking with saturated sodium sulfite and washing twice with aqueous sodium bicarbonate. The organic phase was filtered and dried prior to concentration and separation of products by HPLC as above. The reactions yielded mixtures of the epoxides when analyzed by GC-MS. To obtain the 10,11-epoxide (natural isomer) isomers of JH III farnesal and farnesol we subjected the epoxide fraction collected by HPLC to additional separation using different solvent systems. For example epoxyfarnesal was separated using 5% acetone in hexane, epoxyfarnesol using 10% acetone in hexane and JH III using 15% ethyl acetate in hexane. Individual peaks were collected and analyzed by GC-MS and NMR. Data obtained were compared with spectral data available in the literature or with data generated from the analysis of authentic standards. When authentic standards were not available assignment of epoxide position was deduced based on literature [58]. Mass spectral analysis indicated that all monoepoxides were 90% pure with impurities being small amounts of the 6,7- and 2,3-monoepoxide isomers. Samples of all three of the epoxide isomers of farnesal and farnesol were provided for physiological testing without indicating which sample was the 10,11-epoxide.

BisepoxyJH III was synthesized using the 10,11-monoepoxide isomer of JH III as the starting material. Synthesis was accomplished as above and the products separated by HPLC using 20% ethyl acetate in hexane. This resulted in separation of four fractions that contained di-epoxides of methyl farnesoate. MS and NMR analysis indicated that the naturally occurring isomer eluted last and was ∼75% pure. Therefore, we re-chromatographed this fraction using 15% ethyl acetate in hexane as the eluant. The resulting separation yielded a product that was 96% methyl-6,7; 10,11-bisepoxy-3,7,11-trimethyl-(2E)-dodecenoate, the naturally occurring bisepoxyJH III.

JH III acid was synthesized by saponification of JH III. JH III, dissolved in methanol, was added drop-wise to an equal volume of 2 M KOH and the mixture was stirred overnight at 25 °C. The reaction mixture was neutralized and extracted with ethyl ether. JH III acid was purified by liquid chromatography under gradient conditions using an Adsorbosil C$_{18}$ column (250 × 4.6 mm, 5 mm particles) and detection with a Kratos Spectra Flow 757 variable wavelength detector set at 210 nm. The column was eluted using a linear gradient of 30% acetonitrile to 70% acetonitrile in H$_2$O over 40 min at 1 mL·min^{-1} using a Kratos Spectra Flow 430 gradient former. Under these conditions JH III acid eluted at 24 min and JH III at 33 min. The fraction containing JH III acid was diluted by addition of an equal volume of H$_2$O and extracted with an equal volume of hexane, to remove apolar contaminants, prior to extraction three times with equal volumes of dichloromethane to extract the JH III acid. Analysis of the dichloromethane fraction by GC-MS indicated that the JH III acid was free of JH III. An aliquot of the acid was dissolved in ethanol and derivatized to the methyl ester by addition of an equal volume of hexanes containing 2 M trimethylsilyldiazomethane (Sigma-Aldrich) and stirring for 1 h. Analysis of the derivatized sample indicated that 98% of the acid had been esterified to JH III.

Purification of recombinant USP

Recombinant Trx-his-S-tagged dUSP was prepared essentially as described previously [51]. Briefly, the wild-type or mutant dUSP encoded in the bacterial expression vector pET32EK, in AD 494 bacteria, was induced to be expressed by isopropyl thio-β-D-galactoside. The bacteria were collected, lyzed, and the soluble proteins passed over Ni$^+$-NTA resin (Qiagen, Valencia, CA). Bound dUSP was washed with 10 mM imidazole, and dUSP remaining bound to the resin was eluted with 40 mM imidazole. The eluted dUSP was then subjected to chromatography over a Superdex-200 resin in 50 mM phosphate; 300 mM KCl buffer (pH 7.0) and the fractions containing purified USP combined for fluorescence-binding analysis. The bacterial expression plasmid encoding mutant C472A/H475L was prepared as described previously [48]. The crystal structures of *D. melanogaster* USP dUSP [41] and vertebrate RXR [47] show that the side chains of these conserved cysteine and histidine residues point into the cavity of the ligand-binding

pocket. These two residues do not make contacts with secondary structures or other residues that are necessary for tertiary conformation of the receptor. Nor do the two residues reach the surface of the receptor to make potential contact with coactivators, corepressors, or receptor dimer partners. These two residues are not part of the system of electrostatic contacts that in vertebrate RXR provide a mechanism for allosteric effect of RXR on its receptor dimer partner [59]. Nor are these two residues among those which by mutation cause allosteric effects on hormone binding/transcriptional activation by an ecdysone receptor partner [60]. Finally, both C472 and H475 are located on the secondary structure alpha helix 11, and their mutation to alanine and leucine, respectively, would not be predicted to cause disruption of the helical secondary structure.

Fluorescence-binding assays

Purified Trx-his-S-dUSP was diluted to a final concentration of 300–600 nM in 50 mM phosphate, 300 mM KCl (pH 7.0) buffer. At this receptor concentration the detected intrinsic fluorescence is linear with the amount of receptor protein. The preparation, 4 °C, was excited at 290 nM and fluorescence measured at 340 nm until it had stabilized. Then, either ligand or EtOH carrier was added, and fluorescence was measured. Wild-type dUSP contains only two tryptophan residues (W318; W328), only 10 residues apart on α helix 5 which forms one of the internal borders of the ligand-binding pocket. The side chain of W318 itself is directed into the ligand-binding pocket. Thus, the tryptophan is in a position to potentially sense: (a) static quenching by the ligand; (b) change in its local environment by change in position of amino acid side chains in the neighborhood of the indole group, where the change in local environment happens by either ligand interaction with those neighboring side chains or by ligand-induced movement of secondary structures such as helices; or (c) the net effect on fluorescence of both (a) and (b). Each potential ligand was verified not to significantly absorb fluorescent light at 340 nm (and thereby do not directly absorb and quench the USP intrinsic fluorescence) at the concentrations used. In several cases, the tested ligand did not significantly suppress the fluorescence of the receptor. In such cases, affinity was instead measured by a competition assay: JH III was used (30 μM) to suppress receptor fluorescence, and the test ligand then used over a progressive increase in concentration to compete the JH III from the USP ligand-binding pocket, which then progressively relieved the JH III-induced fluorescence suppression. For all binding data shown, only the 'natural' farnesoid isomer was used.

The binding curves were fitted by nonlinear least squares using an equation in which the free ligand concentration is adjusted for the portion of ligand removed from solution by binding to the receptor [61]. K_d for the interaction of USP with each ligand (or K_i in the cases for competition

assay) was determined from at least three independent binding experiments, except where indicated as being determined from two replications. Variation is shown as standard deviation.

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